Useful Agents for the Study of Glutathione Metabolism in Erythrocytes

ORGANIC HYDROPEROXIDES

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t-Butyl hydroperoxide and cumene hydroperoxide, both known to be substrates for glutathione peroxidase, were used to oxidize erythrocyte GSH. Addition of concentrations of hydroperoxides equimolar with respect to GSH in the erythrocytes or whole blood quantitatively oxidizes GSH in the erythrocytes with a half-time of 4.5 s at 37°C and about three times as long at 4°C. In the presence of glucose, normal erythrocytes regenerate all the GSH in about 25 min. However, glucose 6-phosphate dehydrogenase-deficient erythrocytes failed to regenerate GSH. Treatment of erythrocytes with hydroperoxides does not affect erythrocyte survival in rabbits. Oxidation of erythrocyte GSH with equimolar concentrations of hydroperoxides does not lead to formation of mixed disulphides of haemoglobin and GSH. The hydroperoxides do not affect erythrocyte glycolytic and hexose monophosphate-shunt-pathway enzymes. Previous studies on transport of GSSG from erythrocytes were confirmed by using t-butyl hydroperoxide to oxidize erythrocyte GSH.

Various reagents have been used for the oxidation of intracellular GSH since 1956. At this time it was first demonstrated that primaquine-sensitive erythrocytes were unable to maintain their GSH concentration in the face of the oxidative challenge imposed by incubation with acetylphenylhydrazine (Beutler, 1957). Although this procedure, the glutathione-stability test, proved to be very useful for the detection of glucose 6-phosphate dehydrogenase-deficient erythrocyte, it was less satisfactory for metabolic studies, since both haemoglobin and membrane were extensively damaged during prolonged incubation with this reactive substance. Other com-

presence of glucose. The introduction of methylphenyldiazine carboxylate (azo ester) (Kosower *et al.*, 1965) and diazenedicarboxylic acid bis-(*NN*-dimethylamide) (diamide) (Kosower *et al.*, 1969) provided, for the first time, agents that would very rapidly oxidize GSH stoichiometrically within erythrocytes. These reagents, however, are relatively unstable and may produce extensive cellular damage in addition to oxidizing GSH (Harris & Biaglow, 1972).

We now suggest the use of stable organic hydroperoxides, t-butyl hydroperoxide or cumene hydroperoxide, which rapidly enzymically oxidize erythrocyte GSH by the following mechanism:

pounds, including ascorbic acid, phenylhydrazine and primaquine, which were found to have the same effect (Beutler, 1957, 1960), have the same disadvantage. It was later found that H₂O₂ diffusion would also gradually oxidize the GSH of glucose 6-phosphate dehydrogenase-deficient or glucose-depleted erythrocytes (Cohen & Hochstein, 1961), but although this oxidative challenge is more physiological, many hours of incubation were required and depletion of GSH from normal cells could not be attained in the

(in t-butyl hydroperoxide R = CH₃ and in cumene hydroperoxide R = C₆H₅). t-Butyl hydroperoxide has been used by Sies *et al.* (1972) in studying GSH metabolism of perfused liver. However, we are not aware of any previous efforts to use this class of compounds in metabolic studies of intact erythrocytes. These hydroperoxides exert their effect on erythrocyte GSH by virtue of the fact that they serve as substrates for the enzyme glutathione peroxidase but are not decomposed by catalase.

Materials and Methods

t-Butyl hydroperoxide and cumene hydroperoxide were purchased from Koch-Light, Colnbrook, Bucks., U.K. All the other fine chemicals and the enzymes were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. ^{35}S -labelled GSH was purchased from Schwarz-Mann, Orangeburg, N.Y., U.S.A.

GSH, GSSG and erythrocyte enzymes were determined as described previously (Beutler *et al.*, 1963; Srivastava & Beutler, 1968; Beutler, 1971). However, glutathione peroxidase activity was measured by using 0.1 mM-t-butyl hydroperoxide as substrate instead of H_2O_2 as suggested by Günzler *et al.* (1972). Haemoglobin was determined by using standard procedures and methaemoglobin by the method of Evelyn & Malloy (1938).

Blood from normal and glucose 6-phosphate dehydrogenase-deficient subjects was drawn into heparin and the erythrocytes were washed twice with 7–8 vol. of phosphate-saline (1 part of 0.1 M-sodium phosphate buffer, pH 7.0, plus 9 parts of 0.145 M-NaCl).

Erythrocytes (1 ml) were suspended in 3.0 ml of phosphate-saline containing 5.5 mM-glucose and 1.0 mM-t-butyl hydroperoxide or 1 mM-cumene hydroperoxide. In some experiments 2.0 ml of whole blood was incubated with 2 ml of phosphate-saline containing 1.0 mM-t-butyl hydroperoxide or 1 mM-cumene hydroperoxide. The suspension was agitated in a Dubnoff shaker at 100 oscillations/min at 37°C. Samples were removed at appropriate time-intervals for the determination of GSH and GSSG.

Because the half-life ($t_{1/2}$) of GSH in the presence of hydroperoxides was only 4.5 s it was necessary to stop the reaction almost instantaneously for kinetic studies. Therefore instead of haemolysis of erythrocytes before addition of metaphosphoric acid-EDTA-NaCl reagent as described in the original method for the determination of GSH (Beutler *et al.*, 1963), it was necessary to add the protein-precipitating reagent directly to the erythrocyte suspension. However, addition of the protein-precipitating reagent without prior lysis of erythrocytes resulted in oxidation of a portion of the GSH. Treatment with CO prevented this autoxidation of GSH. To study the $t_{1/2}$ of GSH in the presence of concentrations of hydroperoxides equimolar with respect to GSH, erythrocytes were therefore equilibrated with CO for 5 min before the kinetic studies.

To investigate haemoglobin-GSH mixed-disulphide formation in the presence of diamide and t-butyl hydroperoxide, it was necessary to use haemolysates so that G^{35}SH could be mixed with the haemoglobin. Twice-washed erythrocytes (5 ml) were lysed by freezing and thawing three times and $2\text{ }\mu\text{Ci}$ of G^{35}SH (27 mCi/mmol) was added to the haemo-

lysate. Samples were removed for radioactivity counting in 10 ml of Bray's solution (1960) in a Packard Tri-Carb liquid-scintillation spectrometer and for GSH determination. The haemolysate was divided into three 1 ml portions. One portion was treated with t-butyl hydroperoxide in 0.1 ml of water to make the concentration equimolar with respect to GSH in the haemolysate. The mixture was incubated at 37°C for 1 min. Another portion of haemolysate was treated with diamide in 0.1 ml of water to make the concentration equimolar with respect to GSH. It was mixed and maintained at 4°C as described by Kosower *et al.* (1969). In the third portion of haemolysate, which served as control, 0.1 ml of water was added. In some experiments 0.12 ml of 50 mM-N-ethylmaleimide was added to alkylate haemoglobin thiol groups 1 min after the addition of t-butyl hydroperoxide or diamide to the haemolysate, to prevent the further formation of the mixed disulphide of haemoglobin (Hb) and GSH by the following mechanism:

All the samples were placed on a column (1.5 cm $\times$ 25 cm) of Sephadex G-50 equilibrated with 0.01 M-potassium phosphate buffer, pH 7.0. The samples were eluted at about 35 ml/h and 2.0 ml fractions were collected. A portion (100 μl) from each fraction was transferred into counting vials containing 0.5 ml of Hyamine solution. Bray's (1960) solution (10 ml) was added to each vial and the radioactivity was measured in a liquid-scintillation counter. A portion of each sample was dialysed against 0.01 M-potassium phosphate buffer, pH 7.0, for 4 days with at least two changes of dialysis buffer/day. Samples were taken for measuring the radioactivity.

To study the effect of hydroperoxide and diamide treatment on erythrocyte survival, 15 ml of blood from an albino rabbit, weighing about 4 kg, was centrifuged and the erythrocytes were washed twice with 8 vol. of phosphate-saline. To a 50% (v/v) suspension of erythrocytes in phosphate-saline 0.2 ml of sodium [^{51}Cr]chromate, containing 20 μCi (sp. radioactivity 12.2 mCi/ μmol), was added and the suspension was incubated at 25°C for 25 min. At the end of the incubation, a concentration of t-butyl hydroperoxide or diamide equimolar with respect to erythrocyte GSH was added. The suspension was centrifuged and erythrocytes were washed twice with 5 vol. of phosphate-saline. Samples were removed for the determination of GSH, GSSG and radioactivity. A 50% suspension of these cells was injected into the ear vein of rabbits. One rabbit was used for each reagent. In the control no oxidizing reagent was added to erythrocytes. All the operations were performed under sterile conditions. Blood from each rabbit was drawn at different time-intervals as

described by Beutler & Srivastava (1970), and radioactivity was measured.

To study transport of GSSG from erythrocytes, 1 ml of erythrocytes was incubated for 5 min at 37°C with 3 ml of phosphate-saline containing 1.33 mM hydroperoxide. The erythrocyte suspension was centrifuged and washed once with 8 vol. of phosphate-saline. Erythrocytes (1 ml) were suspended in 3 ml of phosphate-saline and agitated in a Dubnoff shaker at 100 oscillations/min at 37°C. Fluoride (2 mM) or glucose (5.5 mM) was added to the medium where specified. Samples were removed for the determination of GSH and GSSG in the incubation medium and in the erythrocytes. ATP in the erythrocytes was also determined in some cases (Beutler & Mathai, 1967). In some studies washed erythrocytes were incubated for 8 h at 37°C to exhaust them of their endogenous substrates. Such exhausted cells were used for transport studies where specified. ATP in exhausted cells was regenerated by incubation with adenine and inosine before transport studies as described previously (Srivastava & Beutler, 1969b).

Results

Oxidation of GSH to GSSG by hydroperoxides, acetylphenylhydrazine, azo ester and diamide

Addition of a concentration of hydroperoxides equimolar with respect to GSH in the erythrocytes or whole blood quantitatively oxidizes GSH in the erythrocytes with a half-life of 4.5 s at 37°C (Figs. 1 and 2). The half-life of erythrocyte GSH in the presence of *t*-butyl hydroperoxide and cumene hydroperoxide at 4°C was 11.5 and 13.5 s respectively. In the presence of glucose, normal erythrocytes and blood regenerated almost all their GSH in about 25 min (Figs. 1 and 2), whereas glucose 6-phosphate dehydrogenase-deficient erythrocytes failed to regenerate GSH (Fig. 1). The stoichiometry of cumene hydroperoxide oxidation of erythrocyte GSH is presented in Fig. 3. Identical results were obtained when *t*-butyl hydroperoxide was used for the oxidation of erythrocyte GSH.

During oxidation of erythrocyte GSH to GSSG by hydroperoxides or diamide no significant amount of methaemoglobin was formed, whereas treatment with acetylphenylhydrazine or azo ester resulted in formation of up to 30% methaemoglobin.

Formation of haemoglobin-GSH mixed disulphides

During oxidation of erythrocyte GSH by diamide 0.25 mol of GSH was bound as HbSSG per mol of haemoglobin tetramer (Table 1). However, during oxidation of erythrocyte GSH by hydroperoxides only a minimal amount of HbSSG was formed,

representing about one-sixth of HbSSG formed by treatment with diamide (Table 1). This small amount of mixed disulphide is probably formed by the direct reaction between protein and GSSG as described by Srivastava & Beutler (1973).

Effect of various oxidizing reagents on erythrocyte enzymes

To study the effect on erythrocyte enzymes of various reagents used for the oxidation of erythrocyte GSH, 1.0 ml of washed erythrocytes was treated with acetylphenylhydrazine, azo ester, diamide or hydroperoxides. Equimolar concentrations of hydroperoxides were added to the erythrocyte suspension at 37°C as described in the Materials and Methods section and the resulting mixture was incubated for 1 min and chilled in ice. Erythrocyte GSH was oxidized by azo ester and diamide as described by Kosower *et al.* (1965, 1969). A 25% suspension of erythrocytes in phosphate-saline was incubated in a Dubnoff shaker with acetylphenylhydrazine (5 mg/ml of erythrocytes) at 37°C as described by Srivastava & Beutler (1970). Control erythrocytes were not treated with any of the reagents.

Erythrocytes treated with various oxidizing agents and then washed with phosphate-saline were assayed for the following glycolytic enzymes: hexokinase, phosphoglucose isomerase, phosphofructokinase, fructose diphosphate aldolase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerokinase, monophosphate glyceromutase, enolase, pyruvate kinase, lactate dehydrogenase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, glutathione reductase and glutathione peroxidase.

The activity of none of the enzymes studied differed as much as 25% from the control value and the activity of none was decreased more than 12%, although 80–100% of erythrocyte GSH was oxidized by various reagents.

Erythrocyte survival studies in rabbits

Erythrocyte survival studied in rabbits after treatment of erythrocytes with diamide and cumene hydroperoxide was comparable with that in controls. The $t_{\frac{1}{2}}$ of erythrocytes treated with diamide and *t*-butyl hydroperoxide was about 9 days compared with about 8 days for controls.

Transport of GSSG from erythrocytes

The results of transport studies are presented in Table 2. When erythrocyte GSH was oxidized by hydroperoxides, substantial amounts of GSSG appeared in the incubating medium. The rate of

Fig. 1. Oxidation and regeneration of GSH in glucose 6-phosphate dehydrogenase-deficient (a) and normal (b) erythrocytes

Washed erythrocytes (1 ml) were suspended in 3.0 ml of phosphate-saline containing 5.5 mM-glucose and 1.0 mM-hydroperoxide. The samples were incubated at 37°C and at intervals samples were withdrawn for determination of GSH and GSSG. The insert shows the $t_{1/2}$ of GSH in normal erythrocytes treated with t-butyl hydroperoxide as described in the text. Solid lines represent GSH and broken lines represent GSSG. ●, Control; Δ, cumene hydroperoxide; ×, t-butyl hydroperoxide.

transport of GSSG from the erythrocytes was about 50 nmol/h per ml of erythrocytes. The rate of transport remained linear with time for about 5 h; subsequently the rate decreased. This correlated with the decrease in ATP concentrations of the erythrocytes. Transport of GSSG was completely halted if 2 mM-

NaF was added to the incubation medium. The transport of GSSG also halted if before transport studies, erythrocytes were exhausted of endogenous substrates on incubating at 37°C in the absence of glucose for 8 h. At the end of the first incubation for 8 h, erythrocyte-ATP concentrations decreased to

Fig. 2. Regeneration of GSH after treatment of normal blood with hydroperoxides

The experimental conditions were the same as in Fig. 1 except that blood was used instead of erythrocytes. ●, Control; Δ, cumene hydroperoxide; ×, t-butyl hydroperoxide.

about 25% of the normal value. Addition of 10mM-inosine and 1mM-adenine to substrate-exhausted erythrocytes partially restored ATP concentrations, and GSSG transport was also partially restored.

Fig. 3. Oxidation of erythrocyte GSH in the presence of various amounts of cumene hydroperoxide

Washed erythrocytes were equilibrated with CO for 4 min. Samples (0.5 ml) of erythrocytes were incubated with specified amounts of cumene hydroperoxide at 37°C for 1.5 min and assayed for GSH and GSSG.

Table 1. Formation of haemoglobin-GSH mixed disulphides with various reagents

The destromatized haemolysate was incubated with G³⁵SH and t-butyl hydroperoxide and diamide as described in the text.

Expt. no.	Additions to haemolysate		GSH bound to haemoglobin (mol/mol of Hb tetramer)	
	Before incubation	After incubation	Dialysed	Sephadex G-50-treated
1	None	None		0.004
	Diamide	None		0.248
	t-Butyl hydroperoxide	None		0.030
2	None	N-Ethylmaleimide	0.004	
	Diamide	N-Ethylmaleimide	0.188	
	t-Butyl hydroperoxide	N-Ethylmaleimide	0.028	

Table 2. Transport of GSSG from human erythrocytes

Washed erythrocytes were incubated with 3 vol. of phosphate-saline containing 1.33 mM-t-butyl hydroperoxide for 5 min at 37°C, then washed with phosphate-saline to remove excess of hydroperoxide. Samples (1 ml) of these erythrocytes were then incubated at 37°C in a Dubnoff shaker (8 h) with fluoride (2 mM) or glucose (5 mM) as specified and samples were taken for determination of GSSG and GSH in cells and in medium of incubation. ATP was determined in the whole haemolysate (Beutler & Mathai, 1967). ATP-exhausted erythrocytes were incubated with 10mM-inosine and 1mM-adenine for regeneration of ATP.

Erythrocyte preparation	Addition in the medium		ATP (μmol/ml of erythrocytes)	GSSG transported (nmol/h per ml of erythrocytes)
	Glucose (5 mM)	NaF (2 mM)		
1 Normal	0	0	14.42	50
	+	0	14.42	0
	0	+	13.60	0
2 Substrate-exhausted normal erythrocyte	0	0	3.40	2
3 Substrate-exhausted and incubated with inosine (10 mM)+adenine (1 mM)	0	0	13.04	16.5

Discussion

In the past, various reagents have been used to oxidize glutathione in intact cells (Beutler, 1957, 1960; Cohen & Hochstein, 1961; Kosower *et al.*, 1965, 1969). Azo ester (Kosower *et al.*, 1965, 1969), one of the most widely used reagents for intracellular GSH, also oxidizes 30% of haemoglobin to methaemoglobin. Diamide (Kosower *et al.*, 1969), on the other hand, appears to be milder in its action than is azo ester. The amount used for complete oxidation of erythrocyte GSH does not lead to formation of methaemoglobin. However, it also indiscriminately oxidizes thiol groups: Harris & Biaglow (1972) have demonstrated that the amount of diamide required to oxidize all the GSH in Ehrlich ascites-tumour cells, oxidizes about 20% of total protein thiol groups and about 12% of the nicotinamide nucleotides. We found that it results in the formation of mixed disulphides of haemoglobin and GSH in haemolysates.

In the present study we have investigated hydroperoxides, which have been shown to be substrates for glutathione peroxidase (Little *et al.*, 1970), for use in the oxidation of GSH in intact cells. Two hydroperoxides, *t*-butyl hydroperoxide and cumene hydroperoxide, used in this study oxidize erythrocyte GSH by acting as substrate for glutathione peroxidase.

We have compared the effect of acetylphenylhydrazine, azo ester, diamide and *t*-butyl hydroperoxide on various erythrocyte enzymes and methaemoglobin formation. None of the agents had any significant effect on erythrocyte glycolytic and glucose-shunt-pathway enzymes. However, acetylphenylhydrazine and azo ester form substantial amounts of methaemoglobin during oxidation of erythrocyte GSH, whereas diamide or the hydroperoxides did not form any significant amount of methaemoglobin. Oxidation of erythrocyte GSH by diamide or *t*-butyl hydroperoxide did not affect erythrocyte survival in rabbits. It has been pointed out in our previous studies that acetylphenylhydrazine forms significant amounts of haemoglobin-GSH mixed disulphide (Srivastava & Beutler, 1970). Since diamide indiscriminately chemically oxidizes thiols, protein thiol groups or GSH, forming free radicals, it is possible for the free radicals to combine to form mixed disulphides of haemoglobin and GSH according to the following scheme:

A small amount of mixed disulphide may also be formed by direct reaction of GSSG with haemoglobin. In fact, we show that even in less than 1 min at 4°C diamide leads to formation of substantial amounts of mixed disulphides, 0.25 mol of GSH/mol of haemoglobin tetramer. However, the hydroperoxides oxidize GSH in a single enzymic step, by serving as substrate for glutathione peroxidase as described in eqn. (1). Hence, in the presence of concentrations of hydroperoxides equimolar with respect to erythrocyte GSH, no significant quantities of mixed disulphides of haemoglobin and GSH were formed. Only a minimal amount of GSH was found to be bound to haemoglobin in the form of mixed disulphides, probably by the direct oxidation of protein thiol groups by GSSG.

Thus the hydroperoxides, being substrates for glutathione peroxidase, therefore specifically enzymically oxidize GSH, and have an added potential advantage over all the agents used previously as oxidizing agents. Using hydroperoxides, we have confirmed our earlier studies with other oxidative agents (Srivastava & Beutler, 1969a) on the transport of GSSG from erythrocytes.

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